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14. ABSTRACT

Fragile X syndrome (FXS) is the most common inherited form or intellectual disability and a leading genetic cause of autism spectrum disorder (ASD). An expansion of a CGG repeat in the 5' untranslated region of the gene leads to hypermethylation of the gene, and the gene product, FMRP, is no longer made. When FMRP is not made, other genes are improperly regulated, and many of these genes are autism-linked genes. In this project, we have used human-induced pluripotent stem cells (iPSCs) from FXS patients to create human forebrain organoids to study the alterations that occur in human brains when FMRP is absent. We have identified that loss of FMRP in FXS organoids leads to premature differentiation of neural progenitor cells. Further, when we studied the fate of these neurons after differentiation, we identified a significant reduction in GABAergic neurons in the FXS organoids. We also identified an increase in the density of synaptic boutons in the FXS neurons and an increase in the action potential firing frequency, suggesting the hyperexcitability of FXS neurons. When we compared the mRNA targets of FMRP in the FXS organoid to the FMRP targets in mouse cortex, we found that FMRP targets are enriched in neuronal pathways. In particular, the human FMRP targets were significantly enriched among ASD-associated genes. We have also performed RNA-seq analysis of FXS organoids and mouse brains. The differentially expressed genes identified in from FXS organoids were enriched for pathways related to neurodevelopment. These results together show the feasibility of using FXS organoids as a human-specific preclinical model and a resource for studying the molecular pathogenesis of FXS.

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INTRODUCTION: ***Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.***

Fragile X syndrome (FXS) is the most common inherited form or intellectual disability and a leading genetic cause of autism spectrum disorder (ASD). An expansion of a CGG repeat in the 5' untranslated region of the gene to >200 repeats leads to hypermethylation of the gene, and the gene product, FMRP, is no longer made. When FMRP, a protein that is responsible for regulating other genes, is not made, other genes are improperly regulated, and many of these genes are autism-linked genes. In this project, we have used human-induced pluripotent stem cells (iPSCs) from FXS patients to create human forebrain organoids to study the alterations that occur in human brains when FMRP is absent. Substantial progress in characterizing the underlying disease mechanisms in animal models has led to highly successful preclinical studies of drugs modulating metabotropic glutamate and GABA receptors. However, follow-up clinical trials in humans have been largely unsuccessful, highlighting the inexactness of using the mouse model of FXS. We propose to develop and characterize the human forebrain organoids of FXS and identify the human-specific mRNA targets of FMRP during human brain development. The identification of these human-specific targets may reveal druggable targets for both fragile X syndrome and autism in general.

1. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Fragile X syndrome, iPSCs, organoids, autism, FMRP

2. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

1. Establish and characterize FXS organoids and control organoids. (Months 1-9)
2. Use mutant FMRP and isogenic FMR1 CGG deletion lines to confirm phenotypes are due to loss of FMRP. (Months 10-12)
3. Use FMRP CLIP-seq to compare targets between human and mouse samples. (Months 13-16)
4. Examine the RNA modification profiles from human brain organoids from FXS patients. (Months 14-16)
5. Identify the key epitranscriptome alterations associated with FXS that could have functional consequences. (Months 16-18)

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Major Goal 1: Establish and characterize FXS and control organoids.

Organoid lines have been established and characterized. We have compared the lines to determine if loss of FMRP dysregulates human cortical development. We focused on day 56 (D56) forebrain organoids for this comparison. To examine the impact of FMRP loss on neural progenitor cell (NPC) proliferation, we co-immunostained KI67, a proliferation marker, with SOX2, an NPC marker (Fig. 1a). We found a significant reduction of $KI67^+$ cells among all $SOX2^+$ cells within the ventricular zone-like (VZ) structures (Fig. 1b), indicating a reduction in NPC proliferation. NPCs give rise to $TBR2^+$ intermediate neural progenitor cells (IPCs), which differentiate into neurons. We found that while most of $TBR2^+$ IPCs are located at the edge of $SOX2^+CTIP2^-$ ventricular structures in the control forebrain organoids, many of $TBR2^+$ IPCs have migrated out to the $CTIP2^+$ cortical plate-like (CP) layer in the FXS organoids (Fig. 1c&d), suggesting a premature differentiation caused by loss of FMRP. We also observed a dramatic increase in the number of $SOX2^+$ cells escaping from the VZ by crossing the VZ/CP boundary (dash line) into the $CTIP2^+$ CP, further confirming the premature differentiation of FXS organoids (Fig. 1c).

We further assessed neuronal differentiation by quantifying the relative thickness of $SOX2^+$ VZ layer and $CTIP2^+$ (or $MAP2^+$, or $TBR1^+$) CP layer between apical and basal surfaces at day 56 (Fig. 1e). Quantitative analyses showed dramatically reduced VZ thickness but increased CP thickness in FXS organoids (Fig. 1f), likely due to the premature differentiation of FXS NPCs. To confirm the neuronal fates after differentiation, we quantified the ratio of glutamatergic and GABAergic neurons by co-immunostaining of α-CaMKII, which indicates glutamatergic neuronal differentiation, and the GABAergic neuron marker GABA (Fig. 1g). Intriguingly, while the ratio of GABAergic/glutamatergic neurons was about 25/100 in control organoids, the ratio was dramatically decreased to about 7/100 in FXS organoids, indicating a reduction of GABAergic neuronal fate (Fig. 1h). These results revealed that loss of FMRP reduces NPC proliferation, leading to premature neuronal differentiation with reduction of GABAergic neuronal fate.

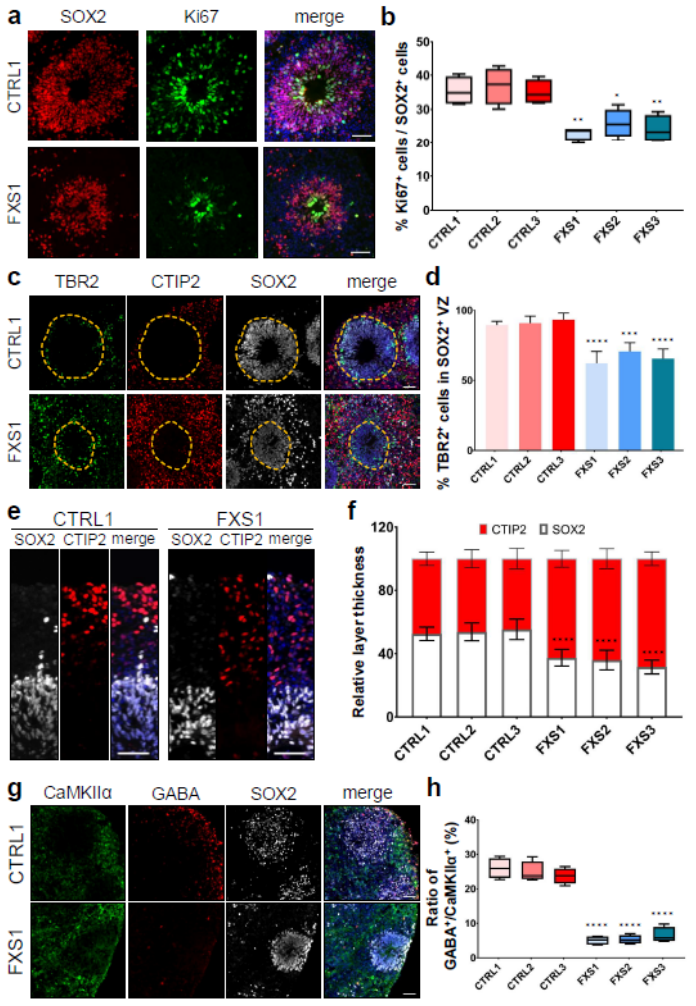

Figure 1. **Loss of FMRP dysregulates neurodevelopment.** (**a-b**) Loss of FMRP reduces NPC proliferation. Shown are representative images (a) and stereological quantification (b) of the proportion of $Ki67^+$ neuronal progenitor cells in both control and FXS-derived forebrain organoids at day 56. Data are presented as mean ± s.e.m. (n = 4 cultures; *P < 0.05, **P < 0.01, one-way ANOVA). Scale bars: 50 µm. (**c-d**) Loss of FMRP dysregulates distribution of $TBR2^+$ intermediate neural progenitor cells. Shown are representative images (c) and quantification (d) of the proportion of $TBR2^+$ IPCs in $SOX2^+$ VZ layer of both control and FXS-derived forebrain organoids. Data are presented as mean ± s.e.m. (n = 4 cultures; ***P < 0.001, ****P < 0.0001, one-way ANOVA). Scale bars: 50 µm. (**e-f**) Loss of FMRP accelerates cortical layer formation. Shown are sample images (e) and quantification (f) of relative thickness of $SOX2^+$ VZ layer and $CTIP2^+$ cortical plate in day 56 forebrain organoids. Data are presented as mean ± s.e.m. (n = 15 sections from 4 cultures; ****P < 0.0001, two-way ANOVA). Scale bars: 50 µm. (**g-h**) Loss of FMRP prevents differentiation of GABAergic interneurons. Shown are sample images (g) and quantification (h) of ratio of $GABA^+$ inhibitory neurons to $CaMKII\alpha^+$ excitatory neurons in both control and FXS-derived forebrain organoids. Data are presented as mean ± s.e.m. (n = 10 sections from 3 cultures; ****P < 0.0001, two-way ANOVA). Scale bars: 50 µm.

We next characterized neuronal function of FXS forebrain organoids. To investigate the impact of FMRP loss on synapse formation, we quantified the density of synaptic puncta by immunocytochemistry of synaptic vesicle protein SYNAPSIN1 (SYN1) and dendrite marker MAP2 (Fig. 2a). The density of SYN1$^+$ synaptic boutons was significantly increased in FXS neurons compared to control neurons in D56 forebrain organoids (Fig. 2b), suggesting an accelerated formation of morphological synapses by FXS neurons.

We next examined the neuronal excitability under current-clamp mode with steps of current injection. Interestingly, we observed a significant increase in action potential firing frequency (Fig. 2c), suggesting the hyperexcitability of FXS neurons. We further characterized the detailed properties of the first action potentials. Quantitative analyses showed significant reduction of decay time of the first action potentials in FXS neurons (Fig. 2d). Furthermore, FXS neurons also displayed larger voltage-activated potassium (K+) currents with normal voltage-activated sodium (Na+) currents ((Fig. 2e-g). These results thus demonstrate that loss of FMRP may lead to neuronal hyperexcitability, potentially caused by the increase of potassium channels in FXS neurons.

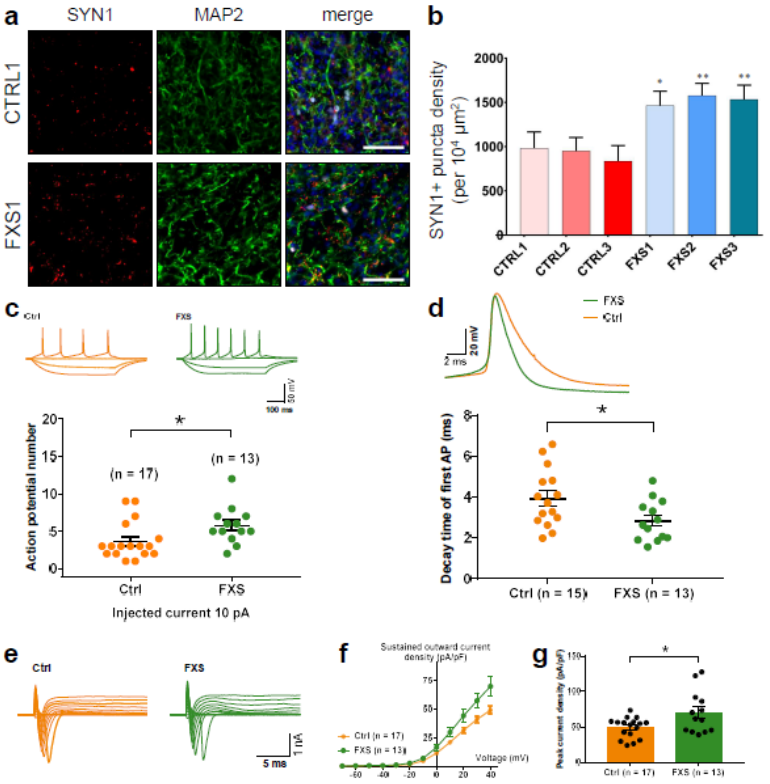

Figure 2. **Loss of FMRP accelerates synapse formation and enhances neuronal excitability.** (**a-b**) Loss of FMRP accelerates synapse formation. Shown are sample images (a) and quantification (b) of SYN1$^+$ puncta density in both control and FXS-derived forebrain organoids at day 56. Data are presented as mean ± s.e.m. (n = 4 cultures; *P < 0.05, **P < 0.01, one-way ANOVA). Scale bars: 50 µm. (**c-g**) FXS forebrain organoids exhibit hyperexcitability. Shown in (c) are sample tracings of action potentials (top) and quantification of action potential frequency (bottom); (d) are sample tracing of the first action potential (top) and quantification of the decay time of first action potential (bottom); (e-g) are sample tracings of transient inward and sustained outward currents (e), quantification of current-voltage curve (f) and peak current density (g). Data are presented as mean ± s.d. (*P < 0.05, **P < 0.01, one-way ANOVA).

Major Goal 2: Use mutant FMRP and isogenic FMR1 CGG deletion lines to confirm phenotypes are due to loss of FMRP.

We will confirm our findings using these cell lines in the last six months of funding, if necessary. At this point, we are seeing a clear, consistent phenotype with the CGG repeat expansion lines. We will continue to focus on characterizing these lines, and if questions arise, we will return to the plan for using mutant FMRP and isogenic CGG deletion lines, if needed.

Major Goal 3: Use FMRP CLIP-seq to compare targets between human and mouse samples.

Given the specific neurodevelopmental deficits associated with the loss of FMRP in human forebrain organoids and more pervasive gene expression alteration in fragile X organoids, we performed enhanced crosslinking and immunoprecipitation followed by high-throughput sequencing (eCLIP-seq) to identify the specific mRNAs that could be bound by FMRP in human forebrain organoids. In comparison, the cortexes of mouse embryonic brain E13.5 were analyzed in parallel. We conducted eCLIP-seq using an FMRP antibody that was used previously for FMRP eCLIP-seq in non-neuronal cells. For all eCLIP-seq experiments, we identified significant peaks by comparing read density between eCLIP and input, excluding PCR duplications and considering only uniquely mapped reads. We used a stringent peak threshold of at least 8-fold enrichment in IP over the input and identified the consistent peaks across the biological replicates.

Gene ontology analysis revealed enrichment for numerous biological pathways involved in neuronal functions. Upregulated genes were involved in nervous system or CNS development, axonogenesis, neuron

projection morphogenesis, axon guidance, protein phosphorylation, neuron projection development, axonogenesis, etc. We next assessed whether FMRP targets are enriched for neuropsychiatric disease genes. We obtained the genes associated with autism spectrum disorder (ASD), schizophrenia (SCZ), depression, aortic lesion, and obesity, and overlapped with FMRP targets of both human and mouse. FMRP targets, particularly the human targets, were significantly enriched among ASD-associated genes. These data are consistent with previous studies showing overlap between genes implicated in neurodevelopmental diseases and FMRP mRNA targets.

Overall our analyses identified more than 3700 mRNAs bound by FMRP in human organoids. Of the 3700 mRNAs, ~1600 overlap with the mRNAs bound by Fmrp in mouse embryonic cortex. ~ 80% of the identified mouse mRNA species are overlapped with the previously published Fmrp mRNA targets in mouse. Of the 2000 human mRNA species, 365 are human-specific and involved in astrocyte differentiation, development, etc. For the mouse-specific RNAs, some of them significantly involved in glutamate receptor signaling pathway. The identification of these human-specific FMRP mRNA targets will provide new insights into the molecular pathogenesis of fragile X syndrome in human context.

Major Goal 4. Examine the RNA modification profiles from human brain organoids from FXS patients.

To determine how the loss of FMRP could impact gene expression during human brain development, we performed RNA-seq using both unaffected and FXS organoids from different developmental stages, D28, D56 and D84, and observed a number of genes that are differentially expressed in the absence of FMRP in a stage-dependent manner. To determine whether the changes that we observed also occurred in human fetal brain, we generated the RNA-seq data using two pairs of human fragile X fetal brain cortex tissues that was collected previously, one full mutation male (post conception week (pcw) 23) and one full mutation female (pcw 24). We observed a significant overlap between differently expressed genes in fragile X fetal brain tissues and FXS organoids of different stages, indicating that FXS organoids could potentially mimic the developmental alterations in human fragile X fetal brain.

Given the significant number of genes with altered expression in FXS organoids, we performed similar RNA-seq analyses using mouse fetal brain of E13.5, which is the similar developmental stage to human D56 organoids. Interestingly we only observed three genes were differentially expressed in the absence of Fmrp in mouse, one of which is the Fmr1 gene. We further examined the genes that display human-specific alteration in the absence of FMRP. These genes are enriched among neurodevelopmental pathways. Together these data suggest that the loss of FMRP could cause a more pervasive gene expression alteration in human brain development.

Major Goal 5. Identify the key epitranscriptome alterations associated with FXS that could have functional consequences.

We will use the remaining funding period to investigate the epitranscriptomic alterations associated with FXS.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to report.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

In the next reporting period, we plan to study the epitranscriptomic alterations in FXS organoids (Major goal #5). We will also use the existing FXS organoid models to test different pharmacologic treatments, including mGluR5 antagonists and PI3K inhibitors.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Development of FXS organoids have the potential of establishing a more human-specific paradigm for understanding the pathogenesis of FXS and potential therapeutic treatments. Previous clinical trials of drugs that have shown efficacy in mouse models, have failed in human trials. Our work has shown that there are many human-specific targets that are not seen in animal models.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to report.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Results from this work are likely to have an effect on clinical trials for FXS. Using a human organoid system enables us to identify the human-specific targets for therapeutic intervention.

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Nothing to report.

that

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Emory University is currently closed to all but essential personnel due to Covid-19. Assuming this closure is not prolonged beyond a few months, we do not anticipate having trouble accomplishing our goals.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required,

were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

The work described above is being prepared for publication.

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series.*

Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

Nothing to report.

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

American Society of Human Genetics Platform Presentation, Houston, TX 2019. Identification of human-specific mRNA targets of fragile X mental retardation protein. Y. Li, Z. Li, Y. Kang, E. Allen, H. Wu, Z. Wen, P. Jin.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report.

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

<u>Example:</u>

Name: *Mary Smith*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID): *1234567*
Nearest person month worked: *5*

Contribution to Project: *Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support: *The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

No change.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*